The mechanism of anticoagulant action of an active anticoagulant fraction (AAF) from the fruits of *M. charantia*

7.1 Brief Introduction

The increasing risk of CVDs has created a niche for the development of a safe, potent, natural, stable anticoagulant drug by the pharmaceutical and/or neutraceutical industries. Nature has been a source of medicinal products for millennia, with many useful drugs developed from plant sources. Exploring our own unexplored medicinal plants might lead to discovery of potent and safe therapeutic agents to treat thrombosis associated cardiovascular diseases.Herbal remedies are often preferred in primary health care because they are cost-effective, culturally acceptable, and potent with fewer side-effects.

The fruit of the bitter melon (*Momordica charantia*), locally known as *Karela*, is an Indian household vegetable, traditionally used in China, India, Africa, and the South-eastern US for treating various diseases [1]. The folk literature of India considers the fruit of *M. charantia* to be useful in treating gout, rheumatism, and sub-acute cases of spleen and liver diseases, malaria, diabetes hepatitis infections and fever [1-4]. In one study, the anticoagulant and fibrin clot-hydrolyzing properties of *M. charantia* seed extracts were explored [5] though no reports have been published on the anticoagulant properties and/or mechanism of anticoagulant action of the whole fruit extract of this plant.

The present study describes a simple method for preparing an active anticoagulant fraction (AAF) from the aqueous fruit extract of *M. charantia*, characterization of the phytochemicals present in AAF, and the mechanism of anticoagulant action of AAF. Further, the antithrombotic effect of AAF was evaluated in the κ-carrageenan-induced mice tail thrombosis model.

7.2 Results

7.2.1 Active anticoagulant fraction (AAF) demonstrated superior anticoagulant and fibrin(ogen)olytic activity, compared to the aqueous extract prepared from the whole fruits of *M. charantia*

The aqueous extract prepared from whole fruits demonstrated superior anticoagulant and fibrin(ogen)olytic activities, in comparison to the activities of the aqueous crude extract prepared from different parts of *M. charantia* (Fig. 7.1a). The anticoagulant

activity of AAF surpasses the activity of the aqueous extract prepared from whole fruits of *M. charantia* (Fig. 7.1b). AAF demonstrated optimum anticoagulant activity at 10 min of pre-incubation with PPP (Fig. 7.1c). The anticoagulant potential of AAF was found to be comparable to heparin or warfarin, though it was significantly less ($p<0.01$) than nattokinase (Fig. 7.1d). AAF significantly prolongs both PT and APTT ($p < 0.05$) of PPP (Fig. 7.1e).

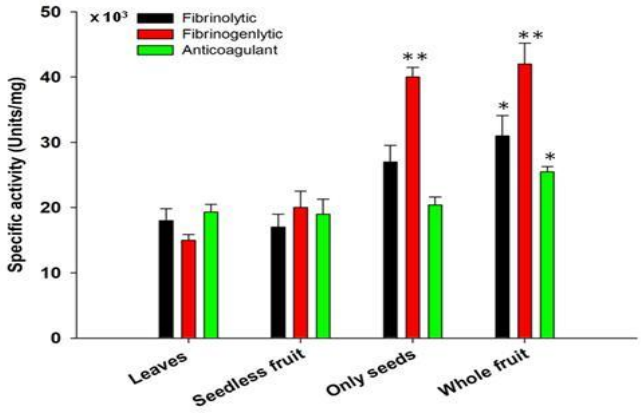

Fig. 7.1a. Comparison of anticoagulant and fibrin(ogen)olytic activity of aqueous extract prepared from seeds, seedless fruits, leaves, and whole fruits of *M. charantia*. Values are mean ± S.D. of triplicate determinations. Significance of difference * $p< 0.05$; ** $p< 0.01$.

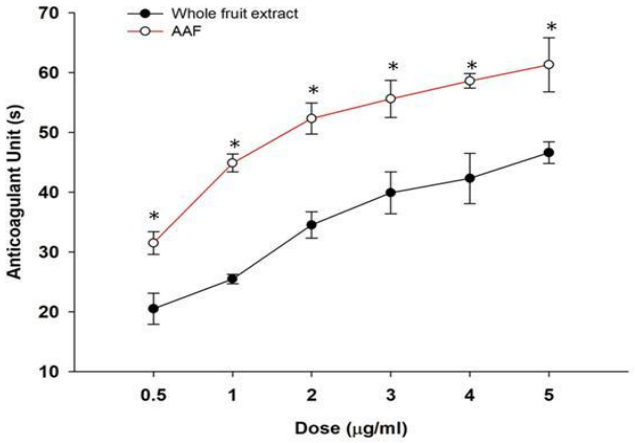

Fig. 7.1b. Dose- dependent *in vitro* anticoagulant activity of AAF and whole fruit extracts of *M. charantia* against goat platelet-poor plasma. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control, * $p< 0.05$; ** $p< 0.01$.

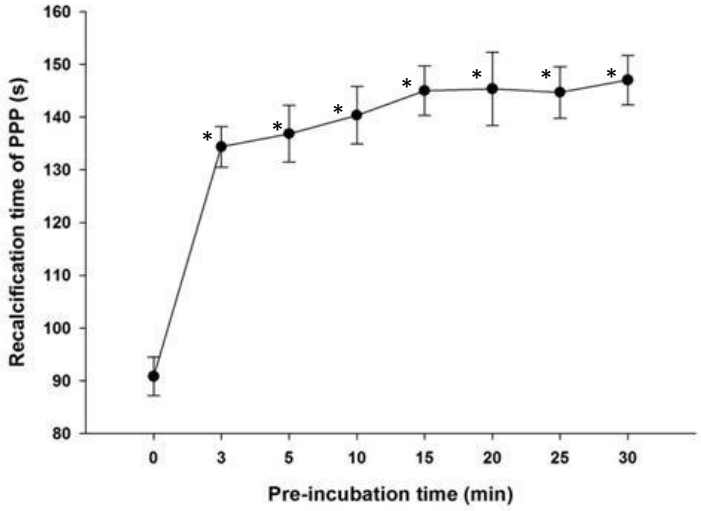

Fig. 7.1c Time- dependent *in vitro* anticoagulant activity of AAF (1.0 µg/ml) against goat PPP. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control (0 min), * p< 0.05.

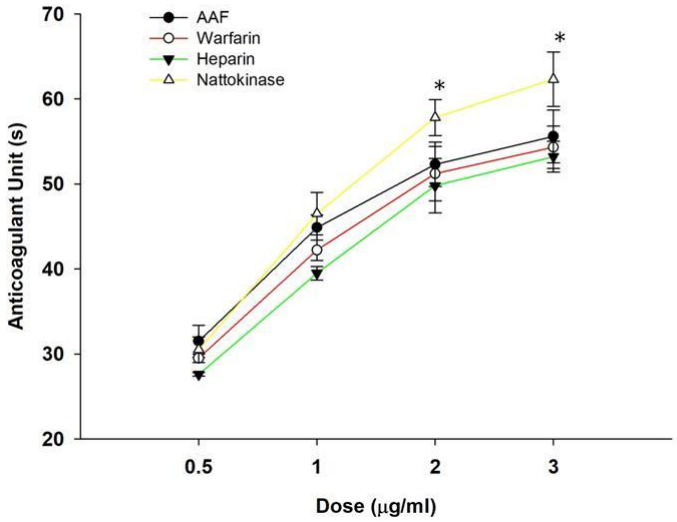

Fig. 7.1d. Comparison of *in vitro* anticoagulant activity of AAF, warfarin, heparin, and nattokinase against goat PPP. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to nattokinase, * p< 0.05.

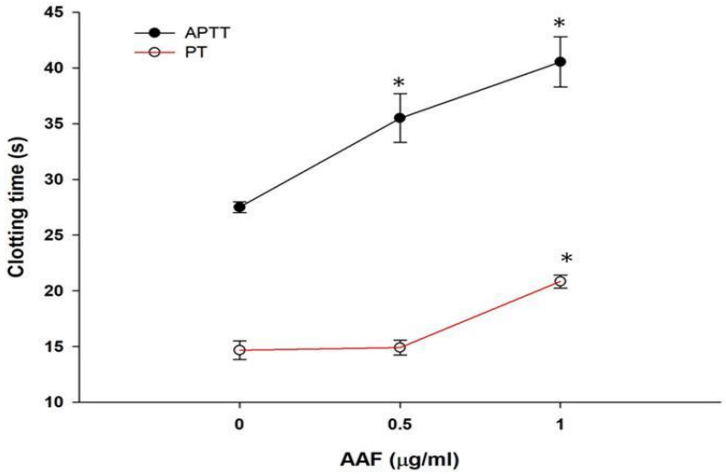

Fig. 7.1e. Effect of AAF (0.5 - 1.0 µg/ml) on APTT and PT of goat PPP. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control, * p< 0.05; ** p< 0.01.

7.2.2 SDS-PAGE analysis of fibrinogen/fibrin degradation by AAF

AAF preferentially degraded the Aα- chain followed by Bβ-chains of fibrinogen; however, the Ɣ-chain of fibrinogen was not degraded even after 3 h of incubation (Figs. 7.2a). The identical result was displayed by nattokinase (Fig. 7.2a). A comparison of fibrinogen degradation by AAF and nattokinase is shown in Fig. 7.2b.

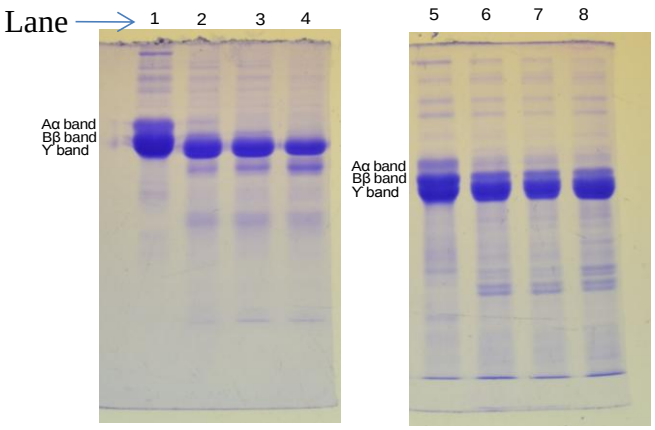

Fig. 7.2a. Kinetics of fibrinogenolytic activity of AAF and nattokinase. The degradation products were separated by 12.5% SDS-PAGE (reducing conditions). Lanse 1 and 5 control human fibrinogen (0.25% w/v in 20 mM K-phosphate buffer, 150 mM NaCl, pH 7.4); lanes 2-4, human fibrinogen treated with AAF (5.0 µg/ml) for 30, 60, and 120 min, respectively at 37ºC, pH 7.4; lanes 6-8, human fibrinogen treated with nattokinase (5.0 µg/ml) for 30, 60, and 120 min, respectively, at 37ºC, pH 7.4.

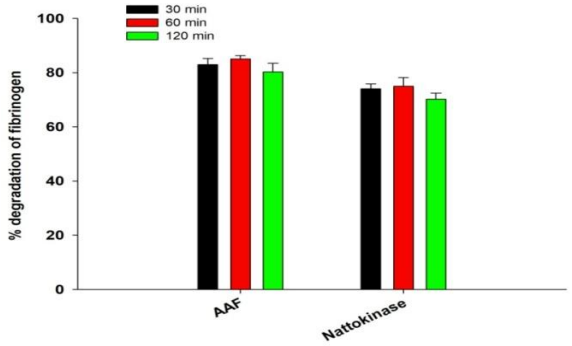

Fig. 7.2b. Densitometry analysis to determine the percent degradation of fibrinogen. All values are means ± S.D. of three independent experiments. p>0.05.

The fibrinolytic activity of AAF and nattokinase is shown in Fig. 7.2c,d.

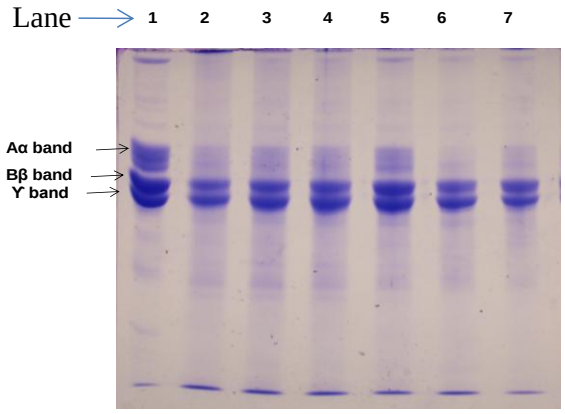

Fig. 7.2c. Kinetics of fibrinolytic activity of AAF and nattokinase. Lane 1, control human fibrin; lanes 2 - 4, human fibrin treated with AAF (5.0 µg/ml) for 30, 60, and 120 min, respectively, at 37ºC, pH 7.4; lanes 5-7, human fibrin treated with nattokinase (5.0 µg/ml) for 30, 60, and 120 min, respectively, at 37ºC, pH 7.4.

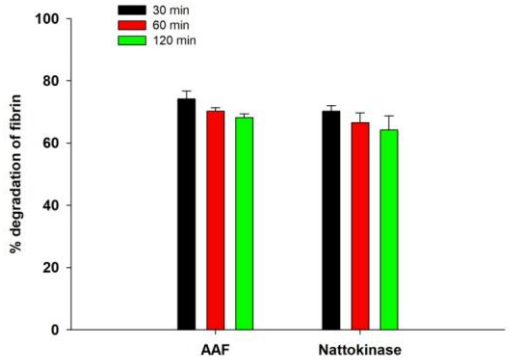

Fig. 7.2d. Densitometry analysis to determine the percent degradation of fibrin. All values are means ± S.D. of three independent experiments. p>0.05

AAF demonstrated a substrate (fibrinogen), concentration-dependent increase in fibrinogenolytic activity, though above a 12.0 µM fibrinogen concentration, no further increase in substrate hydrolysis was observed (Fig. 7.3a). However, after 14 µM substrate concentration, a decrease in fibrinogenolytic activity was observed (Fig. 7.3a). The RP-HPLC analysis of fibrinogen degradation by AAF and nattokinase is shown in Fig. 7.3b.

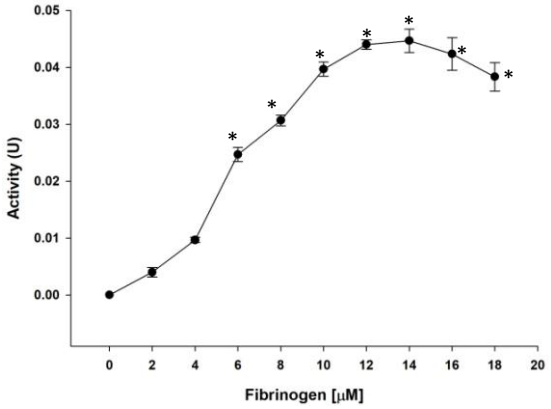

Fig. 7.3a. Effect of substrate (fibrinogen) concentration on protease activity of AAF. The reaction mixture was incubated at 37°C for 60 min with different concentrations (0-18 µM) fibrinogen and the fibrinogenolytic activity was assessed. All values are means ± S.D. of three independent experiments. Significance of difference with respect to control (without fibrinogen), * $p < 0.05$.

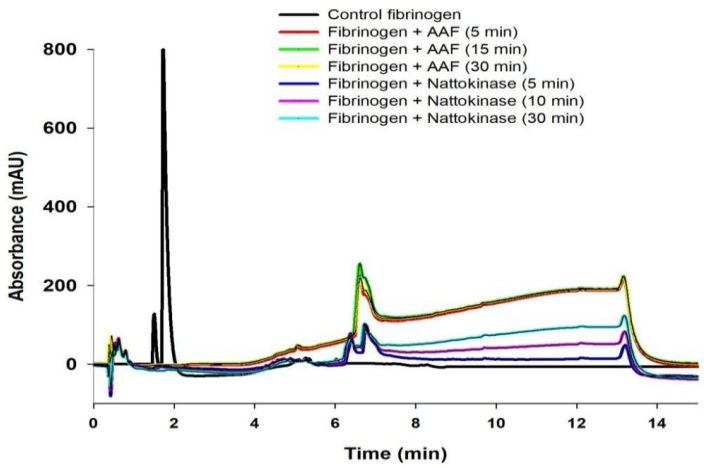

Fig. 7.3b. Comparison of time-dependent fibrinogen degradation between nattokinase and AAF by RP-UHPLC analysis. Fibrinogen was incubated with 1.0 µg/ml of AAF/ nattokinase for 5, 15, and 30 min at 37°C, pH 7.4 and the supernatant was fractionated through an Acclaim® 300 C_{18}RP-HPLC column. For experimental details see section 3.2.7.5.

7.2.3 AAF did not inhibit thrombin or FXa in exerting its anticoagulant action

AAF (1.0-5.0 µg/ml) did not influence amidolytic activity of thrombin (Fig. 7.4a) or change the fibrinogen clotting time of thrombin (Fig. 7.4b). Further, AAF, at a concentration of 1.0 µg/ml did not inhibit the amidolytic activity of FXa against its chromogenic substrate (Fig. 7.4c).

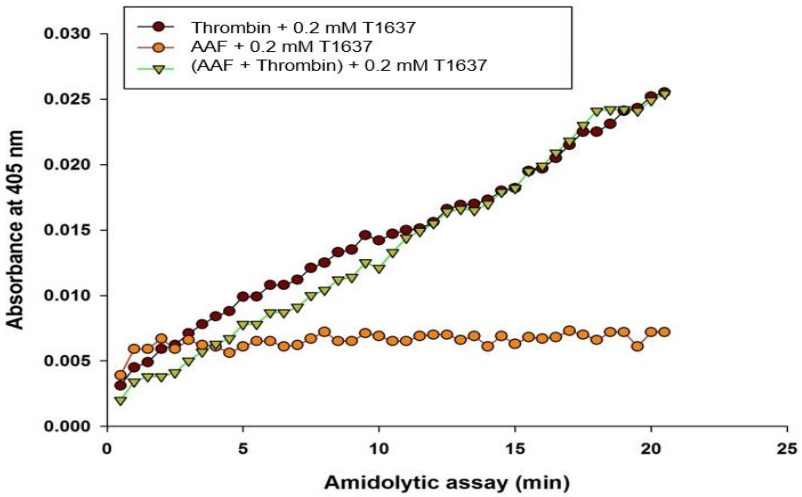

Fig. 7.4a. Effect of AAF (1.0 µg/ml) on amidolytic activity of thrombin (36.6 nM) against its chromogenic substrate T1637 (0.2 mM). AAF does not have any effect on T1637.The plots are the means of three independent measurements.

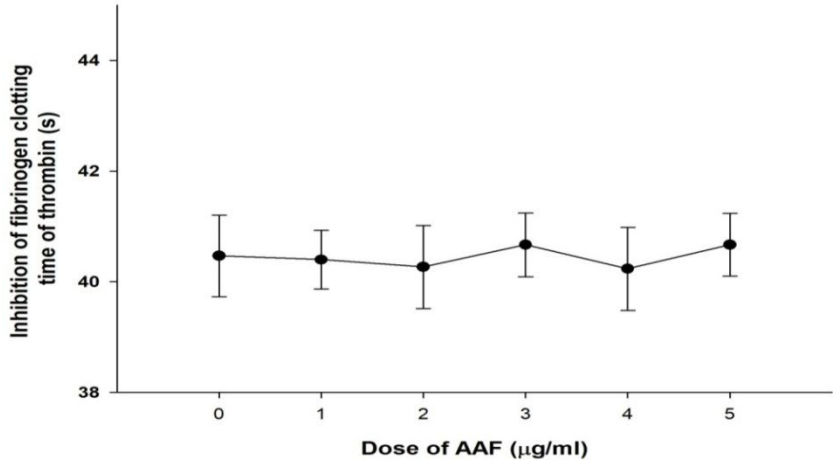

Fig. 7.4b. Effect of AAF (1.0-5.0 µg/ml) on fibrinogen clotting time of thrombin. Values are mean ± S.D. of triplicate determinations.

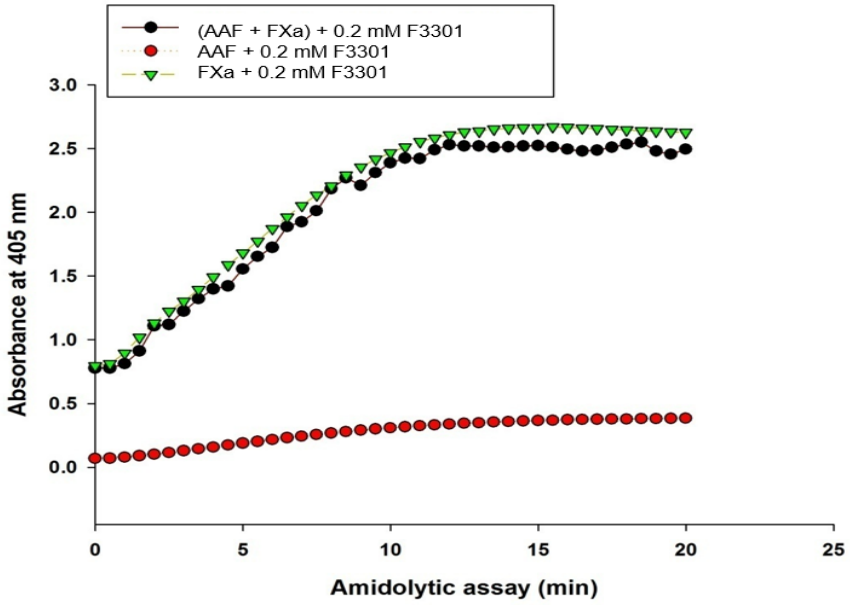

Fig. 7.4c. Effect of AAF (1.0 µg/ml) on amidolytic activity of FXa (1.0 µg/ml) against its chromogenic substrate F3301 (0.2 mM). The plots are the means of three independent measurements.

7.2.4 Fibrin(ogen)olytic activity of AAF was not influenced by endogenous protease inhibitors of plasma but inhibited by serine protease inhibitors

The endogenous protease inhibitors α_2 macroglobulin and antiplasmin did not inhibit the fibrin(ogen)olytic activity of AAF (Table 7.1); however, the fibrin(ogen)olytic activity of AAF was significantly inhibited by serine protease inhibitors PMSF (inhibitor of serine residue), TPCK (inhibitor of chymotrypsin-like serine proteases), TLCK (irreversible inhibitor of trypsin-like serine proteases), and pBPB (alkylation of histidine residue). The protease activity of AAF remained unaffected by IAA (inhibitor of cysteine protease), EDTA (inhibitor of metalloproteases), and DTT (disulfide bond reducing agent) (Table 7.1).

Table 7.1. Effect of chemical inhibitors on fibrin(ogen)olytic activity of AAF. Values represent mean ± SD of three determinations. Significance of difference with respect to control. * $p < 0.01$, ** $p < 0.05$

Inhibitors	**Relative activity (%)**	
	Fibrinogenolytic	**Fibrinolytic**
Control (without inhibitor)	100	100
Inhibitors (concentrations)		
PMSF (5 mM)	65.33 ± 1.3 **	60.33 ± 2.2 **
IAA (5 mM)	98.66 ± 2.7	90.21 ± 3.5
pBPB (5 mM)	2.66 ± 2.1 *	11.50 ± 2.5 *
TPCK (100 µM)	74.0 ± 1.1**	69.50 ± 2.6**
TLCK (100 µM)	41.0 ± 2.3**	33.5 ± 3.8 **
DTT (5 mM)	40.0 ± 3.5**	45.50 ± 5.5 **
EDTA (5 mM)	96.0 ± 5.1	89.33 ± 2.3
α2 macroglobulin	99.8 ± 1.1	98.3 ± 2.3
Antiplasmin	93.22 ± 4.2	90.2 ± 1.8

7.2.5 Spectrofluorometric analysis showed interaction of AAF with fibrinogen

A steady increase in the fluorescence intensity of fibrinogen was monitored in the presence of AAF, suggesting an interaction of fibrinogen with AAF (Fig. 7.5a). No change in fluorescence intensity of fibrinogen was seen in the presence of aqueous rice husk extract, demonstrating the lack of any interaction between rice husk components and fibrinogen (Fig. 7.5b). AAF did not appear to bind with thrombin or FXa (Figs. 7.5c, d)

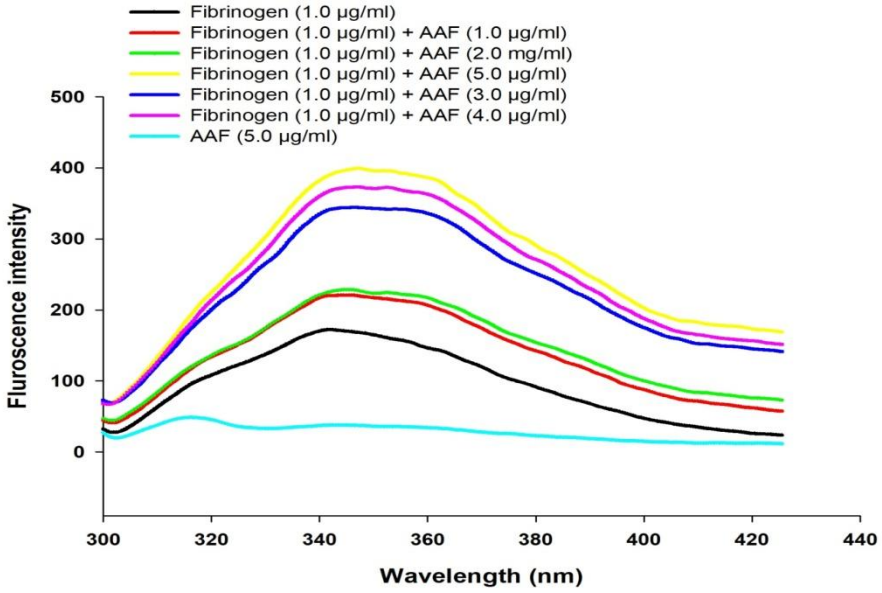

Fig. 7.5a. Fluorescence spectra showing interaction of fibrinogen (1.0 µg/ml) with different concentrations of AAF (1.0-5.0 µg/ml). Data represents the average of five replicates.

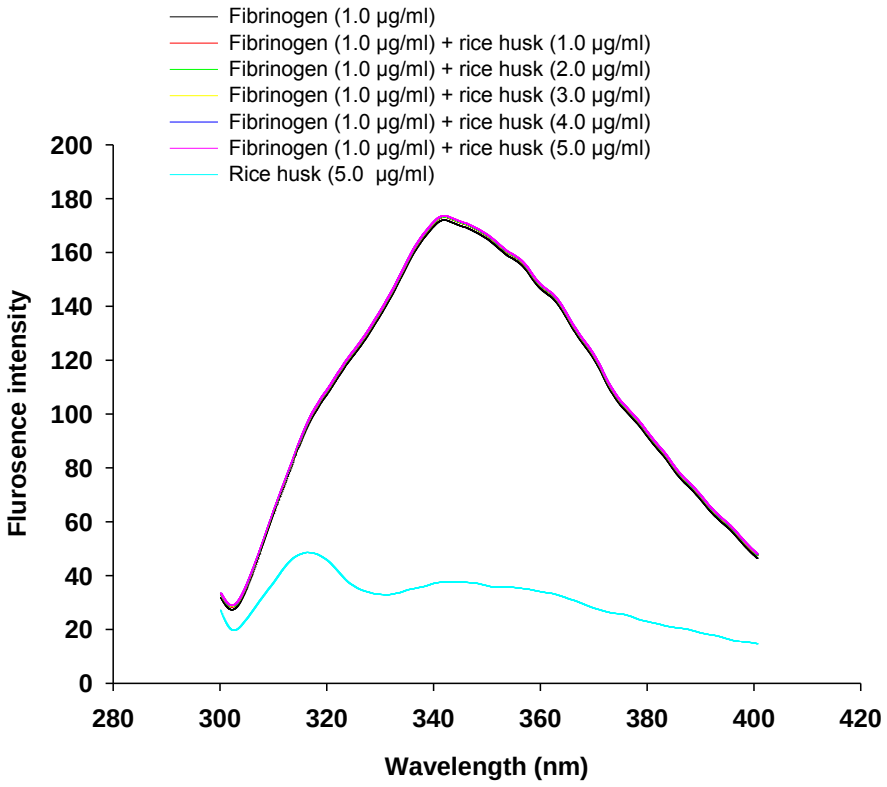

Fig. 7.5b. Fluorescence spectra showing interaction of fibrinogen (1.0 µg/ml) with different concentrations of rice husk (1.0-5.0 µg/ml). Data represents the average of five replicates.

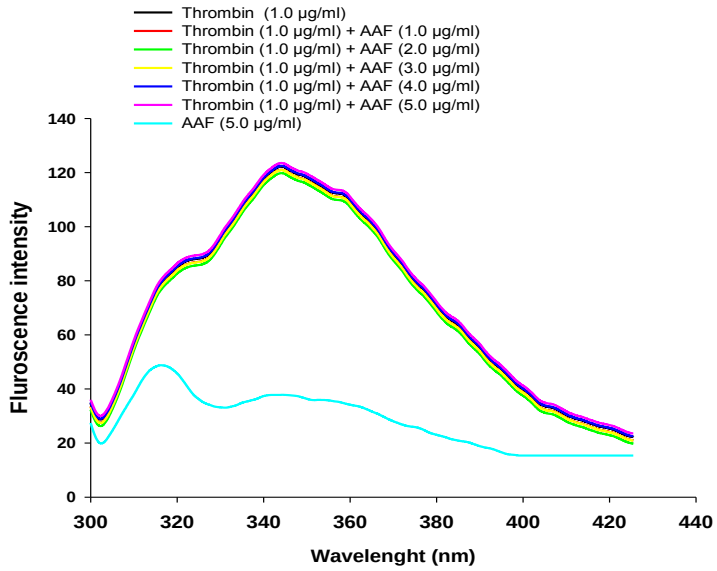

Fig. 7.5c. Fluorescence spectra showing interaction of thrombin (1.0 µg/ml) with different concentrations of AAF (1.0-5.0 µg/ml). Data represents the average of five replicates.

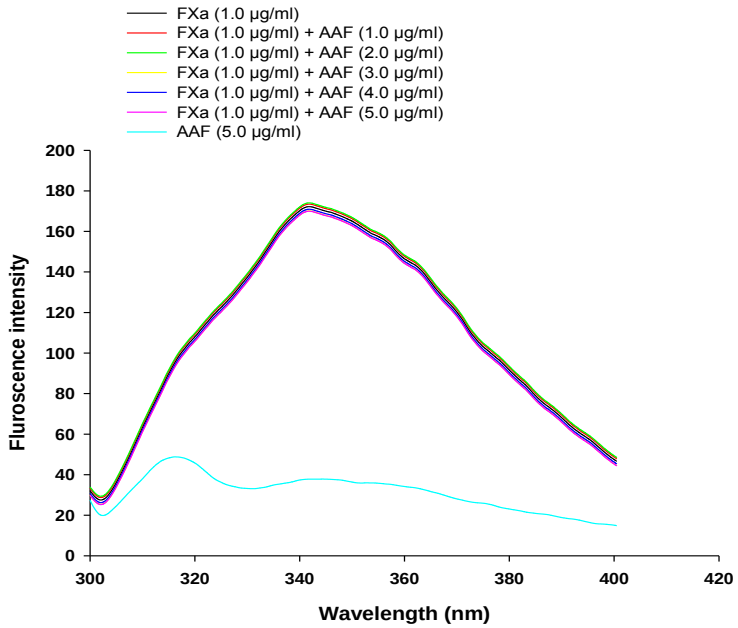

Fig. 7.5d. Fluorescence spectra showing interaction of FXa (1.0 µg/ml) with different concentrations of AAF (1.0-5.0 µg/ml). Data represents the average of five replicates.

7.2.6 AAF demonstrated antiplatelet activity and inhibited collagen and ADP-induced platelet aggregation

A comparison of the dose-dependent platelet de-aggregation (antiplatelet) property of AAF and aspirin (positive control) is shown in Fig. 7.6a. Aspirin showed slightly higher

antiplatelet activity (Fig. 7.6a). AAF also exhibited a dose-dependent inhibition of the collagen and ADP-induced aggregation of PRP (Fig. 7.6b).

a b

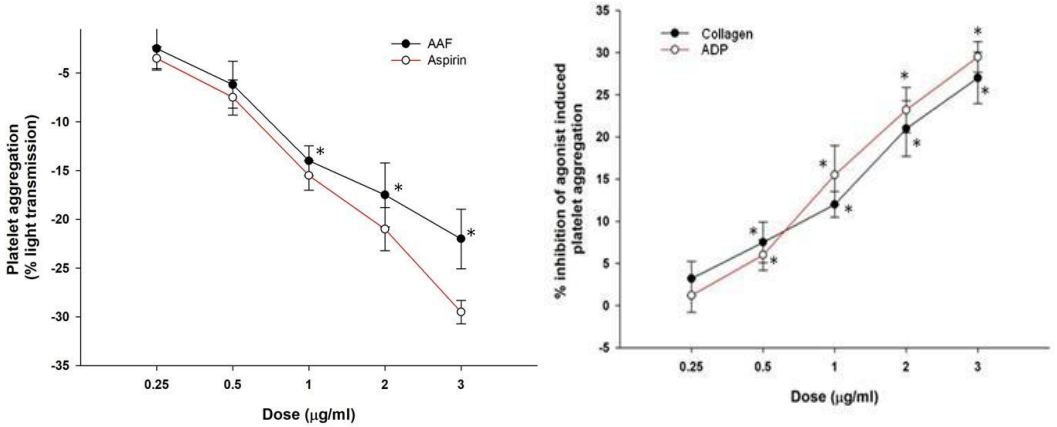

Fig. 7.6a. Dose-dependent platelet deaggregation by AAF/aspirin. Different concentrations (0.25–3 µg/ml) of AAF or aspirin were incubated with platelet rich plasma at 37°C and the absorbance was recorded at 540 nm. **b.** Dose-dependent inhibition of collagen/ADP-induced platelet aggregation by AAF. Different concentrations of AAF (0.25-3.0 µg/ml) were incubated with PRP at 37°C for 10 min and then collagen (6.2 nM)/ADP (30 µM) was added to the reaction mixture. The platelet aggregation induced by collagen and ADP was considered as 100% activity and other values were compared with that. Data represents the mean ± SD of triplicate experiments.

7.2.7 AAF demonstrated *in vitro* clot-bursting activity and an antithrombotic effect but no direct hemolytic activity or cytotoxicity against mammalian cells

A comparison of the thrombolytic potential of AAF and commercial thrombolytic agents is shown in Table 7.2. The *in vitro* thrombolytic activity of AAF was comparable to commercial thrombolytic agent streptokinase and thrombus preventing drug nattokinase (Table 7.2).

Table 7.2. A comparison of the *in vitro* thrombolytic activity shown by AAF, nattokinase, and streptokinase under identical experimental conditions. Values are mean ± S.D. of triplicate determinations. Significance of difference with respect to control, * p< 0.01.

	Mg of clot lysed/µg of sample
A. Non heated blood clot	
Control (1X PBS, pH 7.4)	0.32 ± 0.02
AAF	14.7 ± 0.88
Streptokinase	13.8 ± 0.4
Nattokinase	12.7 ± 1.2
B. Heated blood clot	
Control (1X PBS, pH 7.4	0.27 ± 0.02
AAF	8.5 ± 2.5*
Streptokinase	0.6 ± 0.1
Nattokinase	9.2 ± 1.8*

Pre-incubation of whole blood with AAF resulted in the prolongation of its clotting time in a concentration-dependent manner (Fig. 7.7a). The antithrombotic property of AAF was comparable to nattokinase and streptokinase (p<0.01).

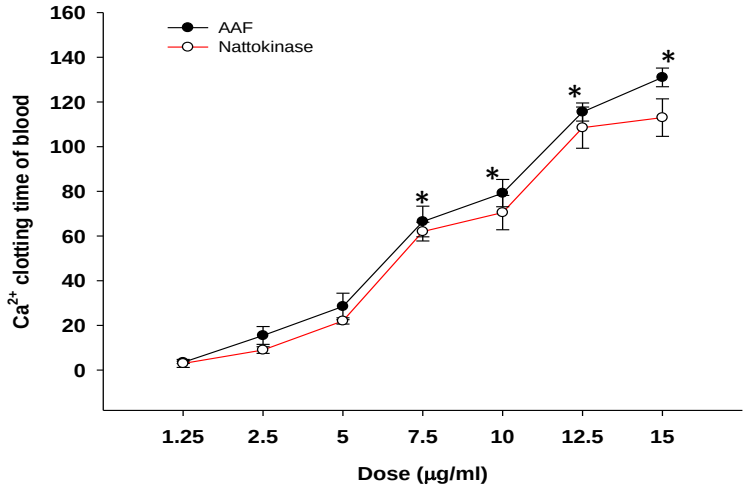

Fig. 7.7a. Dose-dependent (1.25-15 µg/ml) *in vitro* whole blood clotting time of AAF, and nattokinase against mammalian (goat) blood. Significance of difference with respect to nattokinase, * p< 0.05.

AAF, at a concentration of 10.0 µg/ml, did not show adverse effects on the viability of HEK 293 cells (Fig. 7.7b) or any rupturing of mammalian erythrocytes (Fig. 7.7c), indicating its lack of cytotoxicity and haemolytic activity.

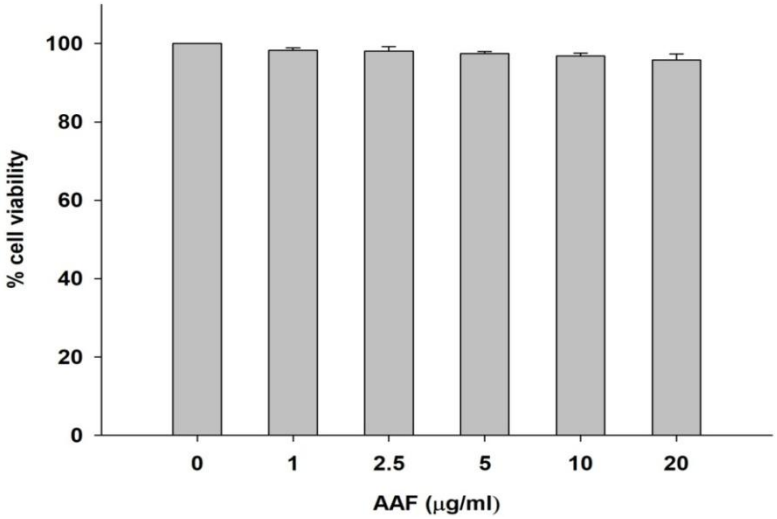

Fig. 7.7b. *In vitro* cell viability assay by MTT assay. HEK 293 cells were treated with AAF (1–20 µg/ml) for 24 h at 37˚C, 5% CO_2. The values are mean ± SD of triplicate determinations.

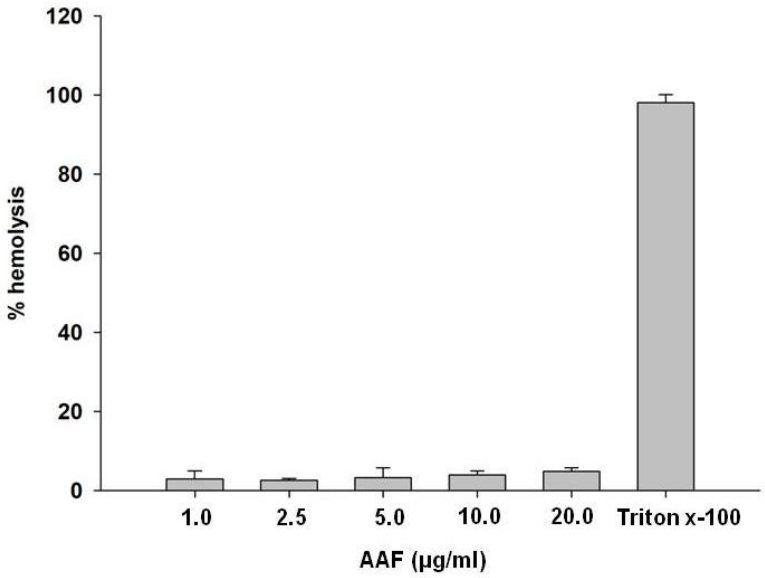

Fig. 7.7c. *In vitro* hemolysis assay. 5% (v/v) erythrocytes obtained from goat blood were treated with AAF (1.0-20 µg/ml) or 0.1% Triton x-100 (100% hemolysis) for 90 min at 37˚C. All values are mean ± SD of triplicate determinations.

7.2.8 Analysis of AAF

7.2.8.1 Phytochemical screening of AAF

Phytochemical screening of AAF revealed the presence of alkaloids, flavonoids, and phytols (Table 7.3). Nevertheless, AAF did not contain glycosides, triterpenoids, tannins, saponins, steroids, or carbohydrates (Table 7.3). The total water soluble extractive from 100 g of dried AAF was found to be 27.4 ± 3.2%. The pH of the 1.0 mg/ml solution of AAF was 6.68.

Table 7.3. Phytochemical analysis of AAF. (+) sign indicates the presence of phytochemicals and (–) sign indicates the absence of phytochemicals.

Phytochemical	AAF
Alkaloid	+
Flavonoids	+
Phytols	+
Glycosides	-
Triterpenoids	-
Steroids	-
Saponnins	-
Tannins	-
Carbohydrates	+

7.2.8.2 Amino acid composition and LC-MS/MS analysis of the protein constituents of AAF

The amino acid composition of AAF is shown in Table 7.4. The SDS-PAGE analysis of AAF showed two major protein bands, with percent composition of 41.8 and 58.2%, of the total proteins of AAF (Fig. 7.8). The molecular masses of the proteins of AAF, which were separated in the upper band, were determined to be ~68-70 kDa, whereas the molecular masses of the proteins in the lower band were found to be in the range of 30-35 kDa. The LC-MS/MS analysis of these two protein bands did not identify protease enzymes in AAF (Tables 7.5 and 7.6).

Table 7.4. Amino acids composition of AAF (% nmol). Values are mean of triplicate determinations.

Amino acids	% nM
Asp	3.94
Glu	3.93
Gly	6.54
Thr	13.13
Cys	2.60
Art	1.23
Ala	2.31
Tyr	5.21
Val	1.33
Met	1.75
Ile	0.86
Leu	1.50
Lys	53.68
Pro	2.00

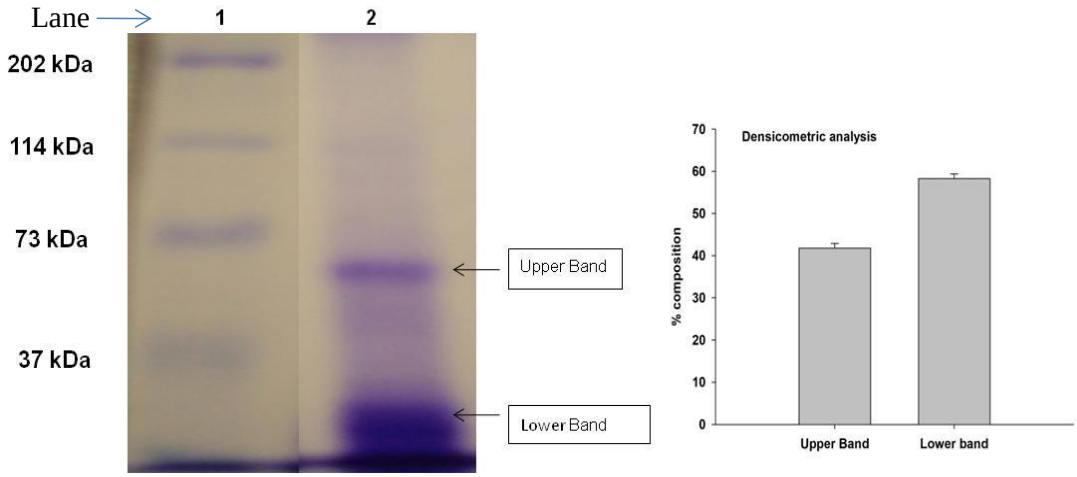

Fig. 7.8. 12.5% SDS-PAGE analysis of AAF. Lane 1, protein molecular markers; Lane 2, reduced AAF (30 µg).Insert. Densitometry analysis of SDS-PAGE protein bands of AAF.

Table 7.5. The proteins identified in the lower and upper SDS-PAG protein bands (see figure 7.8) of the AAF after LC-MS/MS analysis.

S. No.	Accesion No.	Protein	Source of Organism	Score	% Coverage	Unique Peptides	Mass (Kda)
1	gi|952951552	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	*M. charantia*	161.7	12.03	7	84.7
2	gi|973529778	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	*M. charantia*	128.6	8.47	3	45.4
3	gi|218092006	lipoxygenase	*M. charantia*	87.9	4.09	3	99.4

Table 7.6. Peptide sequences detected for the proteins identified by LC-MS/MS analysis.

S. No.	Accession No.	Protein	Score	Peptide sequence	MH+ (Da)	Charge (Ƶ)	Modifications
1	gi|952951552	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	161.7	YGAGIGPGVVYDIHSPR	1658.8	3	
				aSHIVGYPR	1041.5	2	N-Term(Acetyl)

S. No.	Accession No.	Protein	Score	Peptide sequence	MH+ (Da)	Charge (Ƶ)	Modifications
				FLFAGVVDGR	1080.6	2	
				GmlTGPVTILNWSFVR	1806.9	2	M2(Oxidation)
				ALGVDTVPVLIGPVSYLLLSKPAK	2450.5	3	
				ISEEEYVK	996.4	2	
				IVEVNALAK	956.5	2	
2	gi|973529778	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast)	128.6	LSGGDHIHAGTVVGK	1447.7	2	
				VALEAcVQAR	1116.5	2	C6(Carbamidomethyl)
				DNGLLLHIHR	1187.6	2	
3	gi|218092006	lipoxygenase	87.9	HASDEVYLGQR	1274.6	2	
				LYILDHHDALmPYLR	1885.9	3	M11(Oxidation)
				mADFLGNTLK	1125.5	2	M1(Oxidation)

7.2.8.3 GC-MS analysis of AAF

The phytochemicals identified by GC-MS analysis are shown in Table 7.7. The most abundant phytochemicals are decanoic acid, 1,2,3-propanetriyl ester (22.3%), dodecanoic acid, 1,2,3-propanetriyl ester (17.3%) dodecenoic acid, 1,2,3- propanetriyl ester (12.5%), and 4-B-methylandrostane 2,3-diol-1,17-dione (11.4%).

Table 7.7. GC-MS analysis of AAF.

Sl No.	**Phytochemicals**	**Relative abundance (%)**	**Molecular Mass (g/mol)**
1	Decanoic acid, 1,2,3-propanetriyl ester	22.279	639.0019
2	Dodecanoic acid, 1,2,3-propanetriyl ester	17.318	639.00
3	Dodecenoic acid,1,2,3-propanetriyl ester	12.510	611.009
4	4-B-methylandrostane 2,3-diol-1,17-dione	11.418	90.122
5	Trimethylsilyl fluoride	9.623	92.188
6	Propanoic acid, 2-methyl	7.942	88.19
7	Lup-20(29)-en-3-ol, acetate	6.574	468.754
8	Cis-vaccenic acid	5.415	282.468
9	2,6-Bis (3,4-methylenedioxphenyl)-3,7-dioxybicyclooctane	2.712	242.238
10	9,12- octadecadienoic acid	1.747	28.452
11	10- undecynoic acid, trimethylsilyl ester	1.608	198.3019
12	Trans-13- octadecenoic, methyl ester	0.853	296.495

7.2.9 AAF demonstrated post-storage activity

AAF (1.0 µg/ml), post-storage at 30 days at 4°C and at −20°, contained approximately 90% of its anticoagulant (Fig. 7.9a) and fibrinogenolytic activity (Fig. 7.9b). Nevertheless, these activities of AAF showed less stability at room temperature (Figs 7.9a, b).

a b

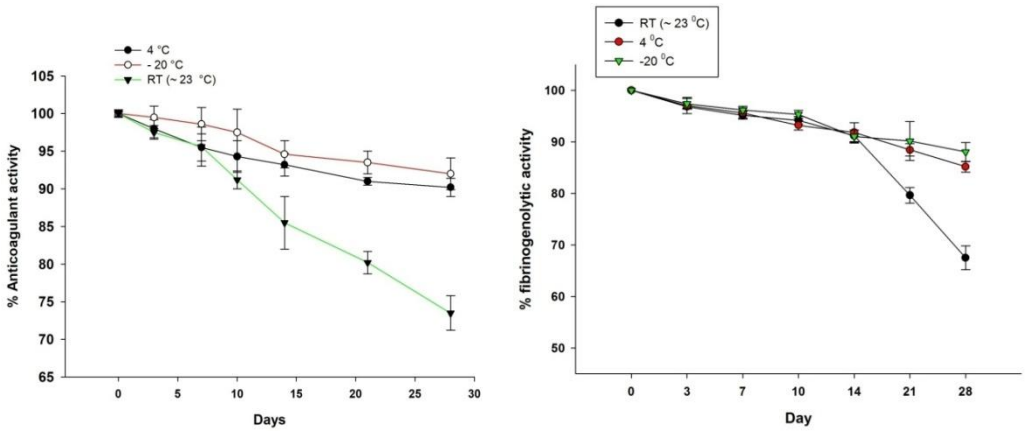

Fig. 7.9 (a) Anticoagulant and (**b)** fibrinogenolytic activity of AAF (lyophilized) at different time intervals and in different storage conditions. The values are mean ± SD of triplicate determinations. Significance of difference with respect to storage at 4 °C and at -20 ºC, *p<0.01

7.2.10 AAF demonstrated *in vivo* anticoagulant and defibrinogenating activity

Tail bleeding time, plasma clotting time, PT, and APTT were significantly (p<0.01) prolonged in AAF-treated mice, in comparison to the control group of mice (Table 7.8). The anticoagulant potency of AAF-treated mice was comparable to nattokinase-treated mice (Table 7.8). AAF demonstrated *in vivo* defibrinogenation of mice plasma (Fig. 7.10). Plasma IgG, IgA, and IgE contents of AAF-treated mice did not differ significantly from those of the control group of mice (Table 7.9).

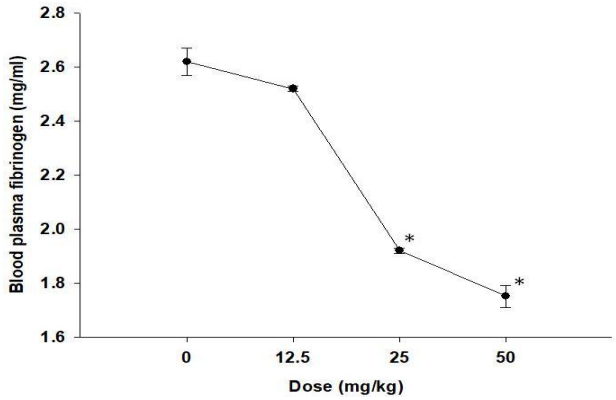

Fig. 7.10. Dose-dependent *in vivo* defibrinogenating activity of AAF 5 h after *i.v.* injection in mice. Values are means ± S.D. of triplicate determinations. Significance of difference with respect to control, *p< 0.05.

7.2.11 Antithrombotic effect of AAF in the mice thrombus model

AAF dose- dependently (12.5 to 50 mg/kg) inhibited thrombus formation in the tail of carrageenan-treated mice (Fig. 7.11 and Table 7.10). The percent inhibition of thrombus formation induced by k-carrageenan in mice tail by AFF and nattokinase is shown in Table 7.11.

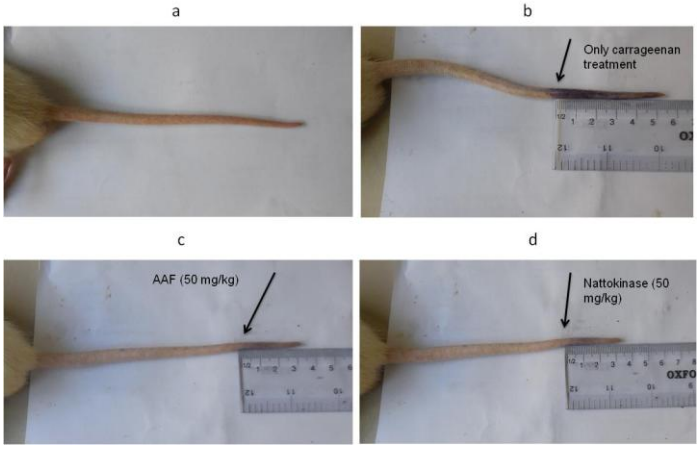

Fig. 7.11. The effect of AAF and nattokinase on carrageenan-induced mice tail thrombus length (48 h after carrageenan injection). **a)** The tail without κ-carrageenan injection, **b)** Control group of mice treated with 0.9 mg/kg carrageenan only, c) AAF (50 mg/kg) pre-treated group of mice injected with 0.9 mg/kg carrageenan, d) nattokinase (50 mg/kg) pre-treated group of mice injected with 0.9 mg/kg carrageenan. Arrows indicate thrombus formation region (wine and black-color). For experimental details, see section 3.2.15.

Table 7.8. A comparison of *in vivo* anticoagulant and defibrinogenating activity of AAF and nattokinase-treated Swiss Albino mice. The blood was withdrawn 5 h after *i.v.* injection of AAF (50.0 mg/kg) or nattokinase (50.0 mg/kg). Values represents mean ± SD of six determinations. Significance of difference with respect to control. *p< 0.01. INR = ($prothrombin_{test}$ / $prothrombin_{control}$).

Drugs (50 mg/kg)	**PT (s)**	**PT (INR)**	**APTT (s)**	**APTT (INR)**	**Tail bleeding time (s)**	**Plasma clotting time (s)**	**Blood plasma fibrinogen (mg/ml)**
1X PBS (control)	14.8 ± 0.7	1.0	28.37 ± 1.4	1.0	45.3 ± 2.5	175.5 ± 9.1	2.52 ± 0.02
AAF	30.5 ± 0.6*	2.05	39.5 ± 2.0*	1.4	108.0 ± 2.6*	209.5 ± 5.7*	1.82 ± 0.03*
Nattokinase	38.6 ± 2.8*	2.60	37.0 ± 3.6*	1.30	120.0 ± 4.1*	211.3 ± 2.8*	1.71 ± 0.02*

Table 7.9. The effect of AAF and nattokinase on k-carrageenan induced mice tail thrombus model at 24, 48 and 72 h post treatment. Values are mean ± SD of six mice. Significance of difference with respect to control group of mice;* p < 0.05, ** p< 0.01

Drugs	**Doses**	**% inhibition of thrombus formation in mice tail after ƙ-carrageenan treatment**		
		24 h	**48 h**	**72 h**
AAF	12.5 mg/kg	9.0 ± 3.1*	13.52 ± 2.5*	21.2 ± 1.2*
	25.0 mg/kg	11.2 ± 2.8*	25.56 ± 3.2*	31.2 ± 2.5**
	50.0 mg/kg	25.4 ± 2.1*	37.00 ± 4.6**	41.3 ± 2.2**
Nattokinase	50.0 mg/kg	26.5 ± 1.2*	46.18 ± 2.75*	52.3 ± 3.8**

7.3 Discussion

In the present study, we developed a simple, one-step cost-effective process for preparing an anticoagulant fraction possessing fibrinogenolytic activity from a whole fruit extract of *M. charantia*. Particular pharmacological activities depend on the nature and polarity of the extraction solvent [6,7]. The bioactive component(s) of *M. charantia* responsible for the anticoagulant activity were more soluble in water than in non-polar solvents; therefore, aqueous extract showed higher activity compared to organic solvent extracts. In addition, therapeutic applications of water-soluble compounds are more promising because of the easier absorption through an oral route [8-10].

The water extract of rice husks (used to prepare AAF) when tested under identical experimental conditions did not show anticoagulant activity, indicating that the rice husk component made no contribution to the anticoagulant activity. The rice-husks served as a good adsorbing agent for any bioactive component(s) of *M. charantia* fruit extract that may have shown anticoagulant activity. To the best of our knowledge, this is the first report showing application of low cost agro waste material rice husk for the preparation of AAF which has a great commercial significance for the development of low cost herbal drug to treat cardiovascular disorder. The proteomics and amino acid composition analyses suggest that AAF contains previously uncharacterized novel plant protease.

Based on their specificity for the α-chain and β-chain of fibrin/fibrinogen, the fibrin(ogen)olytic enzymes are classified as α and/or β fibrinogenases. The α-fibrinogenase from different medicinal plants, such as *Costaria costata* [11] and *Codium fragile* [12] were reported to hydrolyse the Aα-subunit of fibrinogen.AAF contains an αβ-fibrinogenase, because it can degrade both α- and β-chains of fibrinogen/fibrin. Interestingly, an increase in substrate (fibrinogen) concentration (>14.0 μM) resulted in inhibition of the fibrinogenolytic activity of AAF. This observation agrees with reports of the fibrinogenolytic activity of thrombin-like serine proteases such as alborase from *Cryptelytrops albolabris* venom and Russelobin from the venom of *Daboia russelii russelii* decreasing at higher fibrinogen concentrations [13,14]. However, present study shows that the protease present in AAF is capable of degrading higher concentration (12-14 μM) of fibrinogen suggesting its therapeutic application for lowering the fibrinogen level of blood in hyperfibrinogenemia condition.

The significant inhibition of the enzymatic activity of AAF by serine protease inhibitors unambiguously demonstrates that AAF contains a serine protease and lack intramolecular and intermolecular disulfide linkage(s) [14-16]. Failure to inhibit the protease activity of AAF by α_2MG or antiplasmin suggests that this AAF may also exert its activity *in vivo*. This result has a great therapeutic implication because the enzyme after reaching the blood shall not be inhibited and it can exert its function (defibrinogenating activity).

Several mechanisms could explain the anticoagulant action of drug molecules, such as the inhibition of thrombin, FXa; inhibition of platelet aggregation; vitamin k antagonism and/or defibrinogenation of blood plasma [12,17-20]. AAF affected both intrinsic and extrinsic pathways of blood coagulation. AAF does not inhibit thrombin or FXa and exerts its anticoagulant activity via fibrinogenolytic activity. The most abundant phytochemicals suggested by GC-MS/MS analysis are decanoic acid, 1,2,3-propanetriyl ester (22.3%), dodecanoic acid, 1,2,3-propanetriyl ester (17.3%) dodecenoic acid, 1,2,3- propanetriyl ester (12.5%), and 4-B-methylandrostane 2,3-diol-1,17-dione (11.4%). No reports have suggested that these components are present in rice husks; therefore, they are likely components of AAF. Further, none of the above phytochemicals of AAF have shown anticoagulant or antiplatelet activity, though a possible indirect role in enhancing the activity of the fibrinogenolytic enzymes in AAF cannot be ruled out. Although an antithrombin effect of the flavonoids has been reported [21], our results did not show that AAF inhibited thrombin or FXa, suggesting that the flavonoids in AAF do not inhibit thrombin or FXa.

Due to the great sensitivity and selectivity of fluorescence spectroscopy, the powerful tool can be used to study protein-protein interactions [22,23]. The nature of protein-protein interactions may lead to an increase or decrease in the fluorescence [23-25]. In our spectrofluorometric analysis, a steady increase in the fluorescence intensity of fibrinogen was seen in the presence of AAF that suggested the interactions between AAF and fibrinogen.

As explained above, antiplatelet activity is another way to prevent the blood coagulation. AAF shows potent antiplatelet activity and is capable of inhibiting the collagen and ADP-induced platelet aggregation, the precise mechanism of which needs to be explored. Further, the clot bursting activity of AAF was not completely abolished in heat-treated

blood suggesting that this clot lysing action was partially dependent on endogenous factors of blood [19,26]. Usually, patients who develop atrial fibrillation require anticoagulants to prevent the risk of clot formation, which could otherwise initiate cardiovascular diseases [27]. AAF not only delays the progressive coagulation of blood but it also possesses thrombolytic potency, making it a suitable candidate for therapeutic applications as an antithrombotic and thrombolytic agent.

The thrombus forms via the effects of cruor, anticoagulation, the fibrinolytic system, haemorheology, vascular endothelial cells, platelets, and other factors [28,29], and animal thrombosis models are effective for evaluating the activity of thrombolytic agents. As an experimental model of peripheral obstructive disease, the carrageenan-induced thrombosis in mice is used, because of its advantages of being a simple method for inducing thrombus in small laboratory animals and observations are easy without having to kill the animals [28,29]. Our results suggested that AAF can prevent tail thrombosis induced by k-carrageenan and it may be useful as a prophylactic antithrombotic drug.

The oral sub-chronic toxicity studies using fruit extracts of *M. charantia* at a dose of 1g/kg in rats did not show adverse effects or hypersensitivity reaction in rats [2,30-32]. This dose is 400 times greater than the tested dose of AAF (50 mg/kg), showing that the *in vivo* anticoagulant and defibrinogenation activity may be obtainable with a safe administering of the agent having a high therapeutic index. These results should encourage clinical trials of AAF as a possible anticoagulant antithrombotic drug to prevent cardiovascular diseases.

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